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ORIGINAL

DNA extraction from dry wood of *Neobalanocarpus heimii* (Dipterocarpaceae) for forensic DNA profiling and timber tracking

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Abstract Wood can be a good source of DNA for various applications in forensic forestry and timber trade if high-quality DNA can be retrieved from the dry wood. In order to provide a general guideline for DNA authenticity testing established for *Neobalanocarpus heimii*, this study was designed to evaluate the potential of extracting DNA from the dry wood. Overall, the efficacy of DNA extraction was higher for the cambium and sapwood than for the heartwood tissues. In terms of DNA extraction protocols, the Qiagen kit and CTAB with PTB protocol showed higher PCR amplification rates. In order to safeguard the intactness of the DNA, the DNA extraction from dry wood is recommended to be carried out within 6 weeks after felling for logs and 6 months after felling for stumps. The results also showed that the amplicon size might not account for the PCR amplification success rate, and chloroplast genome yielded higher amplification success rate compared with nuclear genome. However, only the chloroplast region can be perfectly retrieved from heat-treated lumber.

Introduction

Neobalanocarpus heimii or locally known as chengal is endemic but widely distributed in Peninsular Malaysia. It is found in diverse localities, on low-lying flat land as well as on hills of up to 900 m (Symington 1943). The species produces a

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naturally, highly durable wood and is among the strongest timbers in the world. It is used for heavy constructions, bridges, boats and buildings, and wherever strength is considered essential (Thomas 1953). Under the IUCN Red List of Threaten Species, it was assigned to the vulnerable category due to a decline in the area of its distribution, the extent of occurrence and/or quality of habitat and actual or potential level of exploitation (Chua 1998). Owing to the high demand for its valuable timber, *N. heimii* is subject to illegal logging and this species might become endangered in the near future.

The use of inbuilt unique properties of DNA within a timber could serve as an important technical element in forensic forestry to support the determination of identity and provenance (Asia Forest Partnership 2005). In combating illegal logging and monitoring forest certification, a chloroplast DNA (cpDNA) marker showing enough geographical structure could be used to differentiate the origin of one source of timber from another (Deguilloux et al. 2003; Tnah et al. 2009), while a highly polymorphic nuclear short tandem repeat (nSTR) marker could be used to generate DNA profiling databases for individual identification, where an illegal log could be matched to its original stump (Tnah et al. 2010a). Herein, the timber can be tracked "from logging to customer." For the moment though, DNA databases for population and individual identification were established for *N. heimii* to serve as DNA authenticity tool for forensic forestry in Peninsular Malaysia (Tnah et al. 2009, 2010a). However, the fundamental challenge to use these DNA track-back systems relies on the possibility to extract DNA from dry wood.

The cross-section of a tree is generally comprised of cambium, sapwood and heartwood tissues. The inner bark, also known as phloem, is the living tissue, which translocates food from the leaves to growing part of the tree. The cambium is composed mainly of living tissue, while the sapwood is composed of both living and dead tissues (Forest Products Laboratory 1999). In the heartwood, all cells are dead and empty, but certain short fragments are still attached on the cell wall (Cano 1996). Numerous studies revealed that extraction from the cambium and sapwood tissues yielded a considerable amount of DNA for PCR amplification (Deguilloux et al. 2002; Rachmayanti et al. 2009). However, only a small amount of DNA was retrievable via heartwood tissue; though, it is the most valuable part of timber.

Extraction of DNA from fresh tissues is routine in studies on tropical forest species, and yet, extraction from dry wood has not been fully explored. Only a few studies have demonstrated the potential for extracting DNA from wood, for instance, robinia (De Filippis and Magel 1998), oak (Dumolin-Lapègue et al. 1999; Deguilloux et al. 2002), *Gonystylus bancanus* (Asif and Cannon 2005) and dipterocarps (Rachmayanti et al. 2006, 2009; Yoshida et al. 2007). Accumulated evidence shows that the extraction of DNA from dry wood will not be straightforward (Deguilloux et al. 2002; Asif and Cannon 2005). In fact, extractions of high-quality DNA and amplification from dry wood samples are hindered by several factors, including inappropriate preservation, exposure to natural degenerative process, microorganism decay and presence of PCR inhibitors; hence, only a small amount of low-quality DNA is retrievable (Lee and Cooper 1995; Cano 1996; Deguilloux et al. 2002; Shepherd et al. 2002). In addition, in many cases of illegal

logging or timber trade, different forms of wood products are seized. For instance, an intact form of log or raw wood is usually seized from a logging concession, while lumber that is supplied in the trading market is processed to the form of sawn or semi-finished wood product, e.g., plywood, veneer, hardboard and beam. All these factors would seem to have a strong effect on the subsequent DNA availability.

In order to provide a general guideline for DNA authenticity testing established for *N. heimii*, the study was designed to evaluate the potential for extracting DNA from logs and stumps after felling and the accessibility of the extracted DNA to nuclear and chloroplast genomes. Specifically, the study aimed at (1) identifying the best DNA extraction protocol from different parts of wood tissues (cambium, sapwood and heartwood), (2) determining the optimal preservation period of wood for DNA extraction, (3) assessing the effect of amplicon size and genome's copy number on the PCR amplification success rate on DNA extracted from wood and (4) evaluating the feasibility to extract DNA from heat-treated lumber.

Materials and methods

Plant material

Two large individual trees (dbh > 30 cm) of *N. heimii* in the Forest Research Institute Malaysia (FRIM) were sacrificed to determine the best DNA extraction protocol from wood. After felling, the logs were placed under a shed while the stumps remained in the field. The DNAs were extracted from the cambium, sapwood and heartwood tissues of both logs and stumps immediately after felling and after keeping for 2, 4 and 6 weeks and 3, 6, 9 and 12 months. To evaluate the feasibility of extracting DNA from heat-treated wood, a small portion of the fresh log was cut into seven pieces of lumber and dried in an oven at 40, 50, 60, 70, 80, 90 and 100°C. As a positive control, DNA was also extracted from leaf samples immediately after felling to infer conformity of nSTR genotypes and chloroplast sequences.

DNA extraction protocol

All DNA extractions were performed under sterile conditions in separate dedicated areas in order to prevent contamination. The surface of leaf and wood tissues was cleaned with diluted bleach. The sapwood and heartwood tissues were drilled using sterile drill bit, and wood shavings were filtered using a sieve. Only, fine wood powders were collected for DNA extraction. Leaf and cambium tissues were homogenized into fine powder with liquid nitrogen using Miller IFM-150 homogenizer (Iwatani).

Three DNA extraction protocols were tested in this study. These were the DNeasy Plant Mini Kit (Qiagen), modified CTAB protocol (Murray and Thompson 1980) and modified CTAB with PTB (*N*-phenacylthiazolium bromide) protocol. Overall, the total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen kit) following the manufacturer's instructions and CTAB protocol following the

procedure described by Murray and Thompson (1980) with modification and further purified using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH). As for the modified CTAB with PTB protocol, the total DNA was extracted using the same procedure of the modified CTAB protocol, except with the addition of 5.0 ml of 0.1 M PTB (Prime Organics) in the extraction buffer. Blank extractions were performed simultaneously, starting with an empty reaction tube containing just extraction buffer, and were treated exactly the same for the rest of the analysis. Total DNA was subsequently quantified using NanoDrop 1000 Spectrophotometer (Thermo Scientific) and 2% agarose gel electrophoresis.

PCR amplification, genotyping and sequencing

A series of PCR primers specific to nuclear and chloroplast genomes was used to test the quality of all DNA samples extracted from leaf and wood tissues. PCR amplification and genotyping were performed for 12 nSTR loci (*Nhe004*, *Nhe005*, *Nhe011*, *Nhe015*, *Nhe018*, *Hbi161*, *Sle392*, *Sle605*, *Slu044a*, *Shc03*, *Shc04* and *Shc07*), which showed specific amplification, single-locus mode of inheritance, absence of mononucleotide repeat motifs and null alleles in *N. heimii* (Tnah et al. 2010b). PCR amplifications were performed in 10 μL reaction mixture, consisting of approximately 10 ng of template DNA, 50 mM KCl, 20 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.4 μM of each primer, 0.2 mM of each dNTP and 0.5 U of *Taq* DNA polymerase (Promega). The reaction mixture was subjected to amplification using a GeneAmp PCR System 9700 (Applied Biosystems), for an initial denaturing step of 94°C for 3 min, 40 cycles at 94°C for 1 min, 45–50°C annealing temperature for 30 s and 72°C for 30 s, followed by 72°C for 7 min. The PCR products were electrophoresed along with GeneScan ROX 400 (Applied Biosystems) internal size standard and run on the ABI 3130xl Genetic Analyzer (Applied Biosystems). Allele sizes were assigned against the internal size standard, and individuals were genotyped using GeneMapper softwares version 4.0 (Applied Biosystems).

PCR amplifications and DNA sequencing were performed for four cpDNA regions, which showed intraspecific variability in *N. heimii* (Tnah et al. 2009): *trn*L intron, *trn*G intron, *trn*K intron and *psb*K-*trn*S spacer. PCR amplifications were performed with 20 μL of PCR reaction mixture, consisting of approximately 10 ng of template DNA, 50 mM of KCl, 20 mM of Tris–HCl (pH 8.0), 1.5 mM of $MgCl_2$, 0.4 μM of each primer, 0.2 mM of each dNTP and 1 U of *Taq* DNA polymerase (Promega). All reaction mixtures were subjected to amplification using a GeneAmp PCR System 9700, for an initial denaturing step of 94°C for 5 min, 30 cycles at 94°C for 1 min, 50–55°C annealing temperature for 1 min and 72°C for 1 min. This was followed by further primer extension at 72°C for 8 min. The PCR products were purified using the MinElute PCR Purification Kit (Qiagen) and sequenced in both directions using the BigDye Terminator Sequencing Kit (Applied Biosystems) based on the standard dideoxy-mediated chain termination method. The sequencing thermal profile was 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min on a GeneAmp PCR System 9700. Sequencing reactions were purified using ethanol precipitation and run on the ABI 3130xl Genetic Analyzer. Sequencing data were

edited and assembled using CodonCode Aligner version 2.0 (CodonCode Corporation).

Results

DNA extraction protocols for cambium, sapwood and heartwood

The Qiagen kit, CTAB and CTAB with PTB protocols yielded average concentrations of 721, 451 and 152 ng/μL of DNA from the cambium, sapwood and heartwood fresh tissues, respectively (Fig. 1). Similarly, after 12 months of preservation, the extraction performed on the cambium tissue yielded higher amounts of DNA, whereas DNA retrieved from the sapwood and heartwood tissues could not be visualized from agarose gel (Fig. 1). Overall, the efficacy of DNA extraction was higher for the cambium and sapwood than for heartwood tissues.

The results for PCR amplification tested on the DNA extracted using the Qiagen kit, CTAB and CTAB with PTB protocols are shown in Fig. 2. Out of a total of 384 separate PCR amplification tests, the CTAB with PTB protocol yielded 88.8% of PCR amplification success rate, while the Qiagen kit and CTAB protocol yielded 78.1 and 69.3%, respectively. In terms of tissue types, the Qiagen kit yielded higher PCR amplification rates from the cambium tissue, while the CTAB with PTB protocol showed higher amplification rates in the sapwood and heartwood tissues.

Optimal preservation period of wood for DNA extraction

Optimal preservation periods of the wood tissues for logs and stumps were studied for 12 months after felling. For logs, results suggested that the DNA extracted from the cambium and sapwood tissues was only well preserved within 6 weeks after felling (Fig. 3). In chloroplast genome, all cpDNA was 100% amplified from the cambium, sapwood and heartwood tissues within 6-week period. Similarly, in

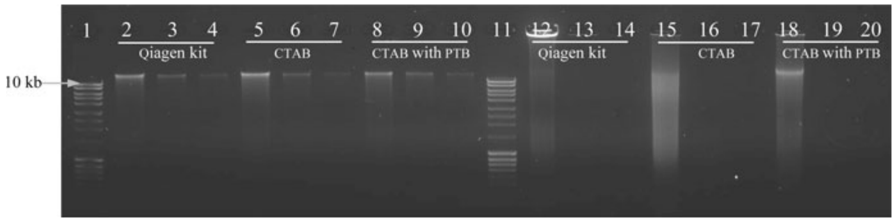


Fig. 1 Agarose gel (0.85%) showing total DNA extracted from the cambium, sapwood and heartwood tissues using three extraction protocols performed immediately after felling: the Qiagen kit (*lane 2* cambium, *lane 3* sapwood and *lane 4* heartwood); the CTAB protocol (*lane 5* cambium, *lane 6* sapwood and *lane 7* heartwood); the CTAB with PTB protocol (*lane 8* cambium, *lane 9* sapwood and *lane 10* heartwood) and after 12 months preservation the Qiagen kit (*lane 12* cambium, *lane 13* sapwood and *lane 14* heartwood); the CTAB protocol (*lane 15* cambium, *lane 16* sapwood and *lane 17* heartwood); the CTAB with PTB protocol (*lane 18* cambium, *lane 19* sapwood and *lane 20* heartwood). *Lanes 1* and *11* denote for MassRuler DNA Ladder (Fermentas)

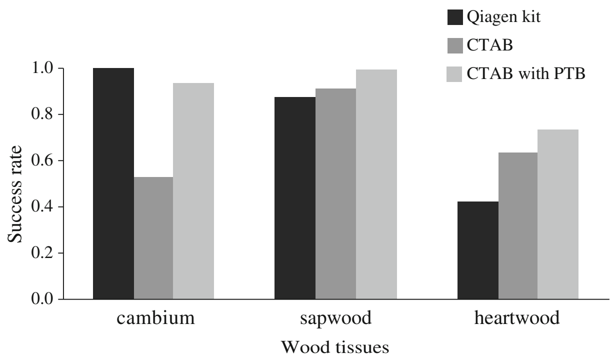


Fig. 2 Three different DNA extraction protocols: the Qiagen kit, CTAB and CTAB with PTB protocol were tested using the cambium, sapwood and heartwood tissues

nuclear genome, all nSTR was only perfectly retrievable from the cambium and sapwood tissues within 6 weeks, while the heartwood yielded inconsistent genotyping results. In contrast, the DNA retrieved via stumps showed better preservation, where the DNA extracted from the cambium tissue was well preserved within 6 months, while that from the sapwood tissue was well preserved within 9 months after felling (Fig. 4). In chloroplast genome, all cpDNA was 100% amplified from the cambium, sapwood and heartwood tissues within 6-month period. In nuclear genome, all nSTR was only perfectly retrievable from the cambium and sapwood tissues within 6 months, while the heartwood yielded inconsistent genotyping results. Overall, the PCR amplification success rates decreased rapidly within 6 weeks after felling for DNA retrieved from logs, while it was 6 months after felling for DNA retrieved from stumps.

Effect of amplicon size and genome's copy number

The effects of amplicon size and genome's copy number on the PCR amplification success rate are shown in Fig. 5. In nSTR, a smaller amplicon size (93–195 bp) yielded almost 100% amplification success rate, while a larger amplicon size (281 bp) yielded 80% amplification success rate. Exception was observed in several amplicon sizes (133–161 bp), which yielded only 70% amplification success rate. On the other hand, the amplification success rate clearly increased as the copy number of the targeted genome increased. For instance, chloroplast genome yielded 90–100% amplification success rate; though, larger amplicon sizes (579–679 bp) were used. Overall, it appears that the amplicon size might not account for the PCR amplification success rate and chloroplast genome yielded higher amplification success rate compared with nuclear genome.

Effect on heat-treated lumber

In heat-treated lumber, only a low quantity of DNA could be recovered (average concentration of 52.77 ng/μl) and it could not be visualized by ethidium bromide

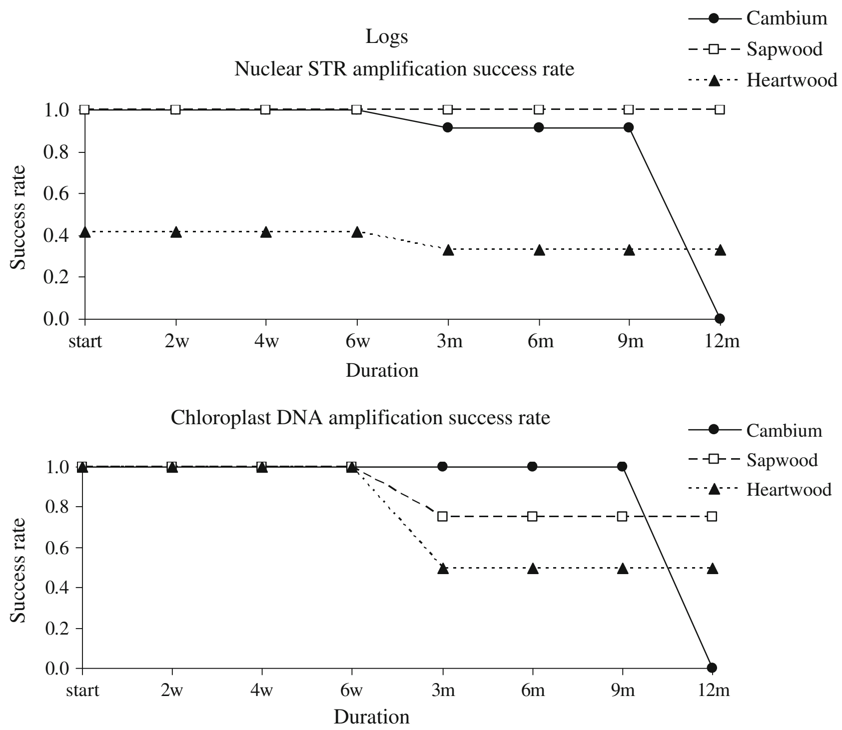
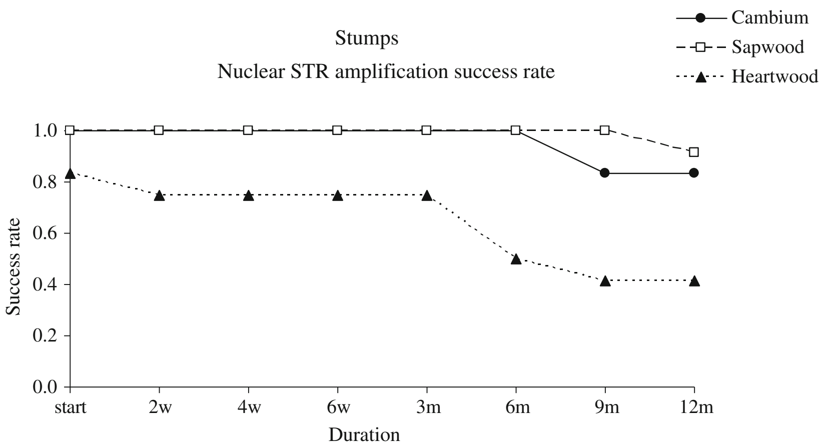


Fig. 3 PCR amplification success rates of DNA extracted from the cambium, sapwood and heartwood tissues for logs. The amplifications were performed for nuclear and chloroplast regions immediately after felling (start) and two (2w), four (4w), six weeks (6w) and three (3 m), six (6 m), nine (9 m) and 12 months (12 m) of preservation

staining of agarose gel (data not shown). Yet, on the basis of the CTAB with PTB protocol, the PCR amplifications showed that both nuclear and chloroplast regions could be retrieved from lumber that was heat-treated at 40–100°C (Table 1). Nevertheless, the phenomena of allelic dropout and inconsistency of genotyping were noted for some of the nSTR regions (Fig. 6). For instance, allele dropout was observed in locus *Nhe011*, where the genotype of control was heterozygote (179/197), while the genotype of heat-treated lumber was found to be in the form of homozygote (197/197). Inconsistency of genotyping was observed in loci *Hbi161*, *Sle392* and *Shc07*.

Discussion

The present study showed that the efficacy of DNA extraction was higher for the cambium and sapwood than for the heartwood tissues. This might indicate that both cambium and sapwood tissues can be a good source of DNA. Similarly, Deguilloux et al. (2002) and Rachmayanti et al. (2009) also reported that the PCR amplification success rates were higher with DNA extracted from the outer part of the logs, from



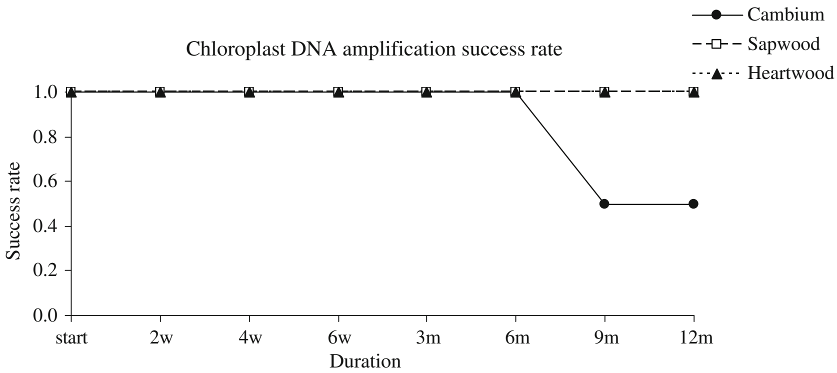


Fig. 4 PCR amplification success rates of DNA extracted from the cambium, sapwood and heartwood tissues for stumps. The amplifications were performed for nuclear and chloroplast regions immediately after felling (start) and two (2w), four (4w), six weeks (6w) and three (3 m), six (6 m), nine (9 m) and 12 months (12 m) of preservation

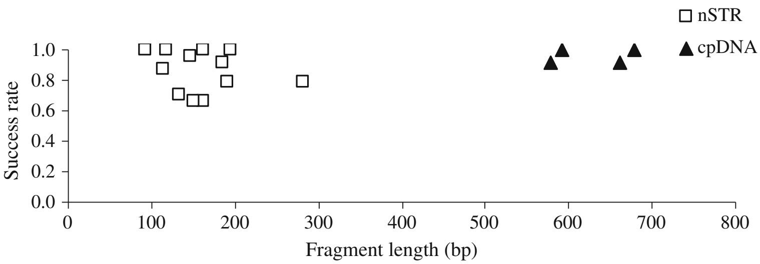


Fig. 5 PCR amplification success rate related to amplified fragment length and genome's copy number (*open squares* indicate nuclear STR, and *black triangles* indicate chloroplast DNA)

cambium to sapwood. The differential of amplification success rates may be simply explained by the gradual transformation of wood cells during aging (Fengel 1970). The cambium is composed mainly of living tissue, while the sapwood is composed

Table 1 PCR amplification tested on DNA extracted from lumber that had undergone drying processes from 40 to 100°C using the CTAB with PTB protocol

Primer	Drying temperature (°C) of lumbers						
	40	50	60	70	80	90	100
Twelve nuclear STRs	+	+	+	+	+	+	+
*trn*L	+	+	+	+	+	+	+
*trn*G	+	+	+	+	+	+	+
*trn*K	+	+	+	+	+	+	+
*psb*K-*trn*S	+	+	+	+	+	+	+

Symbol '+' indicates amplification

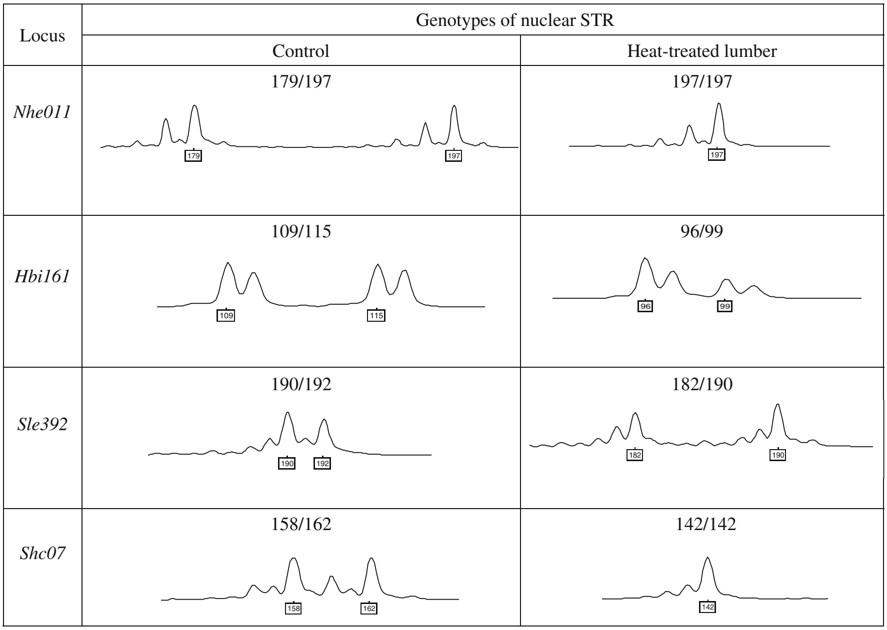


Fig. 6 The problems of allelic dropout and inconsistency of genotyping were observed for DNA amplified from heat-treated lumber using nuclear STR. Genotypes of loci *Nhe011*, *Hbi161*, *Sle392* and *Shc07* for control and heat-treated lumber are shown

of both living and dead tissues. In the heartwood, all cells are dead and empty. Although it is possible to recover DNA from the heartwood, the problems of amplification failure, allelic dropout and inconsistent genotyping might limit the applicability of heartwood tissues for authenticity testing using DNA.

The present study also showed that the Qiagen kit yielded higher PCR amplification success rates in the cambium tissue. The CTAB with PTB protocol showed higher amplification rates in the sapwood and heartwood tissues, while the CTAB protocol generally showed low PCR amplification success rates in all tissue types. Numerous reports have demonstrated the effectiveness of the Qiagen kit to

retrieve DNA from dry wood (Dumolin-Lapègue et al. 1999; Deguilloux et al. 2002; Rachmayanti et al. 2006, 2009; Yoshida et al. 2007); though, Asif and Cannon (2005) have commented that the kit yielded low quality and quantity of DNA. For the CTAB with PTB protocol, the addition of PTB in the CTAB protocol might improve the DNA yields by cleaving sugar-derived protein cross-links and help to release the entrapped DNA (Poinar et al. 1998; Asif and Cannon 2005). The PTB has primarily been used to extract DNA from ancient samples, such as Neanderthal human remains (Krings et al. 1997) and ancient bone (Kelman and Kelman 1999).

The presence of Maillard products in the DNA extracts or impurities such as terpenes, polyphenolics and polysaccharides might also affect DNA quality (Shepherd et al. 2002). Particularly, polyphenolic compounds such as stilbenoids are abundant in the Dipterocarpaceae (Tanaka et al. 2001; Ito et al. 2003) and are believed to be the main PCR inhibitory substances. To improvise the CTAB with PTB protocol, the inclusion of bovine serum albumin in the extraction buffer or alcohol precipitation of DNA in the presence of high NaCl concentration might further enhance the efficiency of polysaccharide and polyphenolic removal (Crowley et al. 2003; Tibbits et al. 2006).

The period of preservation could have a strong effect on the total DNA retrieved from dry wood, as the quality and quantity of DNA are likely to decrease throughout the year. Deguilloux et al. (2002) demonstrated the possibility to retrieve a relatively long fragment of DNA (1,500 bp) from sapwood tissue even after storing for 3 years. However, in this study, the PCR amplification success rate rapidly decreased after 6 weeks of felling for logs and 6 months for stumps. The most likely explanation is that the stumps could be alive for several months and sometimes be able to regenerate into new trees due to the existing root structure. Hence, most of the wood tissues in the stumps are still well preserved; though, exposure of water, UV and microorganisms could probably degrade parts of the DNA molecules (Murmanis et al. 1987; Cano 1996). In contrast to logs, once a tree is cut, the quality and quantity of the DNA in the logs will quickly diminish and more likely be degraded into smaller fragments (Bär et al. 1988; Cano 1996). The optimal period of preservation determined in this study could serve as a useful guide for DNA authentic testing on *N. heimii*. As a whole, for *N. heimii*, in order to safeguard the intactness of the DNA, it is recommended that DNA extraction should be carried out within 6 weeks after felling for logs and 6 months after felling for stumps.

Previous studies reported that the increase in amplicon size would have an adverse effect on the PCR amplification success rate (Pääbo 1990; Deguilloux et al. 2003; Poinar et al. 2003; Rachmayanti et al. 2009). In the circumstance of highly degraded DNA, numerous studies have reported on the poor amplification for the larger amplicon size that ranged between 300 and 500 bp (Wallin et al. 1998; Cotton et al. 2000; Krenke et al. 2002). However, in the present study, there was no obvious relationship between amplicon size and PCR amplification success rate. This might be due to the relatively short fragment length of the nSTR region (93–281 bp) being used in this study. Alternatively, it could be also obscured by differential degradation mechanism of the DNA sequences in a particular genome.

The present study also showed that chloroplast genome yielded higher amplification success rates compared with nuclear genome. By referring to the

PCR amplification from stumps, all cpDNA regions (approximately 600–700 bp) were 90–100% retrievable, while nSTR region only yielded a moderate amplification success rate, although the fragment length of the 12 nSTR is relatively short (approximately 93–281 bp). One possible explanation is that chloroplast genomes are present in multiple copies per cell and greatly more abundant than single-copy nuclear genomes, which leads to higher success rates in amplification.

Drying lumber is one approach of adding value to sawn products from the primary wood processing industries (Haque 2002). In the sawmill, the logs are normally cut into pieces of lumber with various dimensions and subsequently dried at ordinary atmospheric temperatures (air drying) or in a kiln at controlled temperatures raised artificially above atmospheric temperature but not usually above 100°C (Malaysia Timber Council 2006). However, in the context of DNA extraction, heating may lead to the degradation of DNA or inhibition of PCR amplification. Nonetheless, in this study, the PCR amplifications showed that both nuclear and chloroplast regions could be retrieved from lumbers that were heat-treated at 40–100°C, although some of the nSTR regions were embedded with severe allelic dropout problem and inconsistent genotype profile. Likewise, Rachmayanti et al. (2009) demonstrated the possibility to retrieve chloroplast regions from processed dipterocarp wood (either in the form of sawn wood, glued wood or framed wood); though, a lower PCR success rate was observed. In addition, Yoshida et al. (2007) also showed the ability to recover DNA from heat-treated wood from 60 to 140°C. All these might indicate that although heating diminishes the presence of DNA in the wood tissues, a small amount of quality DNA can still be retrieved from heated wood, particularly from chloroplast genome. Thus, the feasibility to retrieve chloroplast region from heat-treated lumber signifies the potential for performing DNA testing for provenance traceability of processed wood.

Conclusion

In summary, the results obtained from this study are ready to be used together with the population and individual identification databases developed by Tnah et al. (2009, 2010a) for timber tracking and forensic DNA profiling of *N. heimii* in Peninsular Malaysia.

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